Cytokine Profiles and Severity of Influenza Infection in Transplant Recipients

Running title: Cytokine profiles in influenza infection

Arnaud G L'Huillier, MD[1], Victor H. Ferreira, PhD[1], Cedric Hirzel, MD[1], Elisa Cordero, MD, PhD[2], Ajit P Limaye, MD[3], Gail Reid, MD[4], Janet Englund, MD[5], Emily Blumberg, MD[6], Deepali Kumar*, MD[1] and Atul Humar*, MD[1]

* Denotes joint senior authorship

[1]Transplant Infectious Diseases and Multi-Organ Transplant Program, University Health Network, Toronto, ON M5G 1J6, Canada;

[2]Hospital Universitario Virgen del Rocío and Biomedicine Research Institute, Seville, 41013, Seville, Spain

[3]Division of Infectious Diseases, University of Washington, Seattle, WA 98195, United States

[4]Loyola University Medical Center, Chicago, IL 60153, United States

[5]Pediatric Infectious Diseases, Seattle Children's Research Institute, Seattle, WA 98105, United States

[6]Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, United States

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Corresponding author:

Deepali Kumar MD MSc FRCPC

Transplant Infectious Diseases

University Health Network

PMB 11-174, 585 University Ave.

Toronto, Ontario M5G 2N2

Canada

Phone: +1-416-340-4241; Fax: +1-416-340-4043

E-mail: deepali.kumar@uhn.ca

ABSTRACT

Influenza is responsible for significant morbidity after transplantation. We evaluated Th1/Th2 cytokines and IL-10 levels during influenza infection in the post-transplant setting. Sera from 277 transplant recipients were analyzed at influenza diagnosis and 28 days later for IFN-γ, IL-4, IL-13 and IL-10. IL-13 levels were associated with protection against pneumonia and ICU admission, whereas the IFN-γ/IL-13 ratio and IL-10 levels were associated with an increased risk of pneumonia and ICU admission. This association was independent of viral load. A skewing of immune responses towards Th2 in transplant patients appears to confer protection from severe influenza infection, independently of viral load.

Key words: influenza; transplantation; outcome; cytokines; hematopoietic stem cell transplantation; solid organ transplantation; immunity; intensive care unit; pneumonia

BACKGROUND

Influenza virus infection is responsible for significant morbidity and mortality in solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) recipients[1]. Despite yearly immunization, a significant proportion of transplant patients develop influenza[1], which is likely related to the underlying immunosuppression, and the discordance between circulating influenza strains and those included in annual vaccine formulations.

Most transplant patients who develop influenza infection will recover fully, although 20-30% develop complications including pneumonia, possibly requiring intensive care unit (ICU) admission or mechanical ventilation, and death[1]. The role that the host immune response plays in the development or prevention of these complications has not been well understood in transplant patients. Also, there is a need for reliable biomarkers to identify transplant patients most at risk for severe infection.

T-helper (Th) immunity is commonly divided into Th1 and Th2. During an infection, Th1 immunity is characterized by induction of opsonizing antibodies, intense phagocytic activity and pathogen clearance, as well as facilitating cell-mediated immunity (CMI) against intracellular microbes via macrophages and CD8 T-cell activation[2, 3]. On the other hand, Th2 immunity assists in the resolution of inflammation and development of humoral immunity against extracellular pathogens[2]. Therefore, the delicate balance between Th1 and Th2 immunity contributes to resolution of infection. The absence of data in the immunocompromised population, combined with the evidence of increased influenza severity in transplant patients, underscores the need to better understand Th1/Th2 responses and IL-10 levels in natural influenza infection, and to evaluate whether cytokine levels can be clinically informative or predictive. Based on prior studies in immunocompetent patients demonstrating worse outcomes of influenza in those with decreased Th2 cytokines and/or elevated IL-10 levels[4-12], we hypothesized that an increased Th1/Th2 ratio and increased IL-10 levels were associated with influenza severity after transplant.

METHODS

Study Design. This study was a sub-study of a multicenter prospective observational study designed as previously described[1]. Briefly, 20 centers in Spain, Canada and United States identified through the American Society of Transplantation Infectious Diseases Community of Practice participated. Local investigators prospectively identified transplant patients with influenza infection. The trial was registered under clinicaltrials.gov (NCT01256255). Inclusion criteria were 1) pediatric or adult SOT or HSCT recipient, 2) microbiologically confirmed influenza by reverse-transcription polymerase chain reaction during the 2010-15 influenza seasons using a nasopharyngeal (NP) swab or bronchoalveolar lavage specimen, 3) informed consent, and 4) available serum specimen at influenza diagnosis. Detailed clinical

information was gathered and patients were followed for six months following influenza diagnosis. The primary and secondary outcomes were pneumonia and ICU admission, respectively.

Cytokine Analysis. Blood was taken at enrollment and 28 days later. Blood was centrifuged for 10 minutes at 2,000x g, and collected serum was stored at -80ºC. IFN-γ was selected as the Th1-representative cytokine, IL-4 and IL-13 as Th2-representative cytokines, and IL-10 as an immunoregulatory cytokine. Specimens with insufficient sample volume for accurate cytokine measurement were excluded (n=14). Cytokine analysis was performed using the Luminex™ 100 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, AB, Canada). All analyses were run in duplicate. The quantification limit (QL) was 0.01pg/mL for IFN-γ, IL-10 and IL-13, and 0.01ng/ml for IL-4. For the purposes of statistical analysis, values below the QL were given a value of half the QL.

Viral Load Analysis. NP swabs were performed at day 0, 3, 6,11, 18 and 28 after enrollment for viral load (VL) quantification in influenza A-infected patients, as previously described[1].

Statistical Analysis. The Th1/Th2 ratio and IL-10 levels were compared between patients with or without pneumonia or ICU admission using Mann-Whitney U-test. Post-hoc analysis compared IFN-γ and IL-13 levels between patients with or without complications using Mann-Whitney U-test, and correlated VL half-life and cytokine values using Spearman correlation. To adjust for multiple comparisons, Bonferroni adjustment was performed and p-values <0.0125 were considered significant. Statistics were performed using SPSS version 23.0 (IBM Corp., Armonk, NY). Figures were made using GraphPad 7.0 (LaJolla, CA).

RESULTS

Demographics

Of the 616 patients with influenza infection included in the overall cohort, 277 (45.0%) had sera available at enrollment. Of these, 229 also had day 28 sera available. The clinical and epidemiologic characteristics of our subcohort were similar than those of main cohort[1] and are shown in Table 1. All patients were treated with neuraminidase inhibitors.

Cytokine levels at enrollment and at day 28

Cytokine values were analyzed at enrollment and day 28. All 277 patients had quantifiable IFN-γ (median 0.82pg/mL [IQR 0.64-1.42]) and IL-13 (median 4.86pg/mL [IQR 1.23-33.07]) at enrollment (Supplementary Figure 1A-B). IL-10 was

quantifiable in 92.1% (255/274) of patients at enrollment, with a median value of 1.39pg/mL (IQR 0.05-5.92) (Supplementary Figure 1C) whereas IL-4 at enrollment was quantifiable in 27.1% (75/269) with the median value below the QL. At day 28, all 229 patients had quantifiable IFN-γ (median 0.74pg/mL [IQR 0.61-1.01]) and IL-13 (median 6.26pg/mL [IQR 1.25-33.15]). IL-10 was quantifiable in 86.8% (196/228), with a median value of 0.07pg/mL (IQR 0.01-1.47), whereas IL-4 was quantifiable in only 21.6% (49/227) with a median value below the QL (Supplementary Figure 1A-C).

Role of immunosuppression and immunization status on cytokine levels are described in Supplementary results.

Th1/Th2 ratio

Based on the hypothesis that the balance of Th1 vs Th2 responses is important in influenza pathogenesis, we analyzed the IFN-γ/IL-13 ratio since both cytokines were detectable in all patients. The range of Th1/Th2 ratios at baseline is shown in Supplementary Figure 1D (median 0.26; IQR 0.06-0.75). Significant variation was observed with some patients showing a skewing towards a Th1 response and others towards a Th2 response. By day 28, the median Th1/Th2 ratio was slightly lower than at baseline (median 0.18; IQR 0.05-0.61) (Supplementary Figure 1D).

Association of cytokines and Th1/Th2 ratio with disease severity

We evaluated the association of cytokine levels, as well as Th1/Th2 ratio, with influenza severity, defined as the presence of pneumonia at presentation (n=62) or the need for ICU admission (n=28). First we analyzed day 0 and day 28 IFN-γ and IL-13 values in patients with and without pneumonia and ICU admission. At both time points, we found no differences in IFN-γ levels between patients who did or did not have either pneumonia or ICU admission (Supplementary Figure 2A-B). In contrast, IL-13 levels at day 0 and day 28 were lower in patients with pneumonia and in those with ICU admission, although this was only significant at day 28 (Supplementary Figure 2C-D).

We then evaluated the Th1/Th2 profile (IFN-γ/IL-13 ratio) and observed that a lower Th1/Th2 ratio at day 0 was associated with decreased incidence of ICU admission, whereas a lower Th1/Th2 ratio at day 28 was associated with a decreased incidence of both pneumonia and ICU admission (Figure 1A-B).

We assessed whether IL-10 levels were associated with disease severity by comparing levels between patients with and without severe influenza. Higher levels of IL-10 at day 0 were associated with an increased risk of ICU admission, and higher levels of IL-10 at day 28 were associated with an increased risk of both pneumonia and ICU admission (Figure 1C-D).

Mortality data related to cytokine levels are described in Supplementary results.

Viral load

VL was tested in the 187 patients. Median VL at influenza infection onset was 4.25log copies/ml (IQR 3.05-5.52log copies/ml) and median time to clearance was 6 days (IQR 3-11). There was no correlation between baseline VL (p=NS for all comparisons), viral clearance kinetics (as determined by VL half-life) and either Th1/Th2 ratio (Correlation Coefficient: 0.071; p=0.454) or IL-10 levels (Correlation Coefficient: -0.108; p=0.259) at day 0.

DISCUSSION

This study evaluates Th1/Th2 responses during influenza infection in a large cohort of transplant patients. The delicate balance of Th1/Th2 responses are likely to play an important role in the pathogenesis and recovery from infections[2, 3]. The main finding of our study was that patients with immune responses directed towards Th2 had a lower incidence of pneumonia or ICU admission, as reflected by IL-13 levels but also by the IFN-γ/IL-13 ratio. This association was independent of baseline VL. Both IL-4 and IL-13 are prototypical Th2 cytokines that stimulate the humoral immune responses during an infection[2]. IL-4 was undetectable in most patients of our cohort, confirming previous findings in influenza-infected immunocompetent children[8], and thus not used for group comparisons. The protective effect of Th2 cytokines during influenza infection is debated, some studies in immunocompetent cohorts having shown a protective effect[7, 11], others a deleterious effect[5, 6] or no association between Th2 cytokines and influenza severity[4]. Differences in studies could be related to different cytokine assays, use of antivirals, different times between symptom onset and blood collection, or the presence of a possible viral or bacterial co-infection[4-7, 11].

IFN-γ was quantifiable in all our patients, although levels were lower than in influenza-infected immunocompetent cohorts[4-6, 9, 10, 13]. Previously, studies have shown that IFN-γ levels are higher in influenza-infected patients compared to controls[4, 10, 13]. Surprisingly, the interpatient variation in IFN-γ levels was low and levels did not correlate with influenza severity, which is consistent with most studies performed in immunocompetent cohorts[5, 9, 13]. The fact that IFN-γ seems protective in some series[7, 10, 11] and deleterious in others[6] is likely related to its typical proinflammatory profile which increases pathogen clearance but also increases the risk of immunopathology[3]. These differences might also be explained by the fact that some studies only investigated H1N1/pdm09-infected patients, whereas others included H3N2- or influenza B-infected individuals, the latter being associated with higher IFN-γ levels[9].

The other important finding of our study was the relationship between elevated IL-10 levels and pneumonia, ICU admission, and late mortality. IL-10 is produced by many cell types of the innate and adaptive immune system and is the most potent anti-inflammatory cytokine[14]. By inhibiting macrophages, Th1 and natural killer cells, IL-10 reduces tissue damage but also impairs pathogen clearance in bacterial, viral and protozoal infections[14]. Thus, IL-10 is frequently

considered a double-edged sword. When compared to healthy controls, IL-10 levels were increased in influenza-infected patients[4, 10, 13]. The relationship between IL-10 levels and influenza severity has been extensively studied in immunocompetent populations. Although few studies were unable to demonstrate any relationship[7, 13], most have associated IL-10 levels with influenza severity[4, 8-10, 12], including pneumonia and ICU admission[5, 6, 9]. Although those studies were performed on immunocompetent cohorts, results are consistent with our data showing that IL-10 levels are higher in patients with pneumonia and ICU admission, especially evident at day 28, despite levels in our cohort being lower than in influenza-infected immunocompetent individuals[4-6, 8-10]. The strong anti-inflammatory effects of IL-10 leading to reduced pathogen clearance could explain why elevated levels were associated with influenza severity. Alternatively, vaccine studies have shown dysregulated IL-10 production in elderly vaccine non-responders[15]; similar to older adults, transplant patients have impaired CMI and a dysregulated IL-10 production during natural infection could impair development of immunity, therefore increasing disease severity.

This study has some limitations. First, this is a substudy of a larger study; however, it is still the largest study of immune response to influenza after transplantation. Second, although there was heterogeneity in type of transplant, there were no significant difference in cytokine levels between HSCT and SOT patients, nor between organ transplants. Also, due to the study design, it is not possible to confirm whether cytokines determined at influenza diagnosis are the cause or the consequence of influenza severity. Finally, IL-4 levels were undetectable in most patients, and therefore this cytokine could not be used for Th1/Th2 ratio.

In conclusion, this is the first study examining serum cytokines during natural influenza infection in a large cohort of transplant patients. Despite levels of cytokines significantly lower than in immunocompetent cohorts, our data showed that an immune response directed towards Th2 was associated with decreased influenza severity and IL-10 was associated with increased influenza severity. These associations were independent of VL, highlighting that the nature of the immune response strongly contributes to disease severity. Further studies are needed in immunosuppressed cohorts to evaluate whether these cytokines could have clinical utility as biomarkers to predict influenza severity or to help guide modification of immunosuppression in patients with severe influenza.

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References

1. Kumar D, Ferreira VH, Blumberg E, et al. A Five-year Prospective Multi-center Evaluation of Influenza Infection in Transplant Recipients. Clin Infect Dis **2018** Apr 7. doi: 10.1093/cid/ciy294. [Epub ahead of print].

2. Spellberg B, Edwards JE, Jr. Type 1/Type 2 immunity in infectious diseases. Clin Infect Dis **2001**; 32:76-102.

3. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol **2004**; 75:163-89.

4. Bradley-Stewart A, Jolly L, Adamson W, et al. Cytokine responses in patients with mild or severe influenza A(H1N1)pdm09. J Clin Virol **2013**; 58:100-7.

5. Matsumoto Y, Kawamura Y, Nakai H, et al. Cytokine and chemokine responses in pediatric patients with severe pneumonia associated with pandemic A/H1N1/2009 influenza virus. Microbiol Immunol **2012**; 56:651-5.

6. Takano T, Tajiri H, Kashiwagi Y, Kimura S, Kawashima H. Cytokine and chemokine response in children with the 2009 pandemic influenza A (H1N1) virus infection. Eur J Clin Microbiol Infect Dis **2011**; 30:117-20.

7. Wong SS, Oshansky CM, Guo XJ, et al. Severe Influenza Is Characterized by Prolonged Immune Activation: Results From the SHIVERS Cohort Study. J Infect Dis **2018**; 217:245-56.

8. Kim YH, Kim JE, Hyun MC. Cytokine response in pediatric patients with pandemic influenza H1N1 2009 virus infection and pneumonia: comparison with pediatric pneumonia without H1N1 2009 infection. Pediatr Pulmonol **2011**; 46:1233-9.

9. Lee N, Wong CK, Chan PK, et al. Cytokine response patterns in severe pandemic 2009 H1N1 and seasonal influenza among hospitalized adults. PLoS One **2011**; 6:e26050.

10. Yu X, Zhang X, Zhao B, et al. Intensive cytokine induction in pandemic H1N1 influenza virus infection accompanied by robust production of IL-10 and IL-6. PLoS One **2011**; 6:e28680.

11. Zhang YH, Wang DY, Wang S, et al. [The analysis of effect of Th1/Th2 cytokine in the different prognosis in severe influenza A (H1N1)]. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi **2011**; 25:274-6.

12. Oshansky CM, Gartland AJ, Wong SS, et al. Mucosal immune responses predict clinical outcomes during influenza infection independently of age and viral load. Am J Respir Crit Care Med **2014**; 189:449-62.

13. Heltzer ML, Coffin SE, Maurer K, et al. Immune dysregulation in severe influenza. J Leukoc Biol **2009**; 85:1036-43.

14. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. J Immunol **2008**; 180:5771-7.

15. Corsini E, Vismara L, Lucchi L, et al. High interleukin-10 production is associated with low antibody response to influenza vaccination in the elderly. J Leukoc Biol **2006**; 80:376-82.

Supplementary results

Role of immunosuppression on cytokine levels

Among our SOT patients, there was no significance difference in IFN-γ, IL-10, IL-13 or IFN- γ/IL-13 ratio at day 0 and day 28 between the 187 patients treated with calcineurin inhibitors but no mTOR inhibitors and the 15 patients treated with mTor inhibitors but no calcineurin inhibitors (data not shown).

Association between cytokine levels and mortality

There was no association between IFN-γ, IL-13 or IFN-γ/IL-13 ratio at day 0 with mortality at day 28 (data not shown). There was no association between IL-10 levels at day 0 with mortality at day 28. However, IL-10 levels were higher both at day 0 (6.18pg/ml [IQR 1.33-35.17] vs 1.29pg/ml [IQR 0.04-5.70]; p=0.004) and day 28 (3.13pg/ml [IQR 0.54-32.76] vs 0.05pg/ml [IQR 0.01-1.42]; p<0.001) among non-survivors at 6 months.

Role of immunization status on cytokine levels

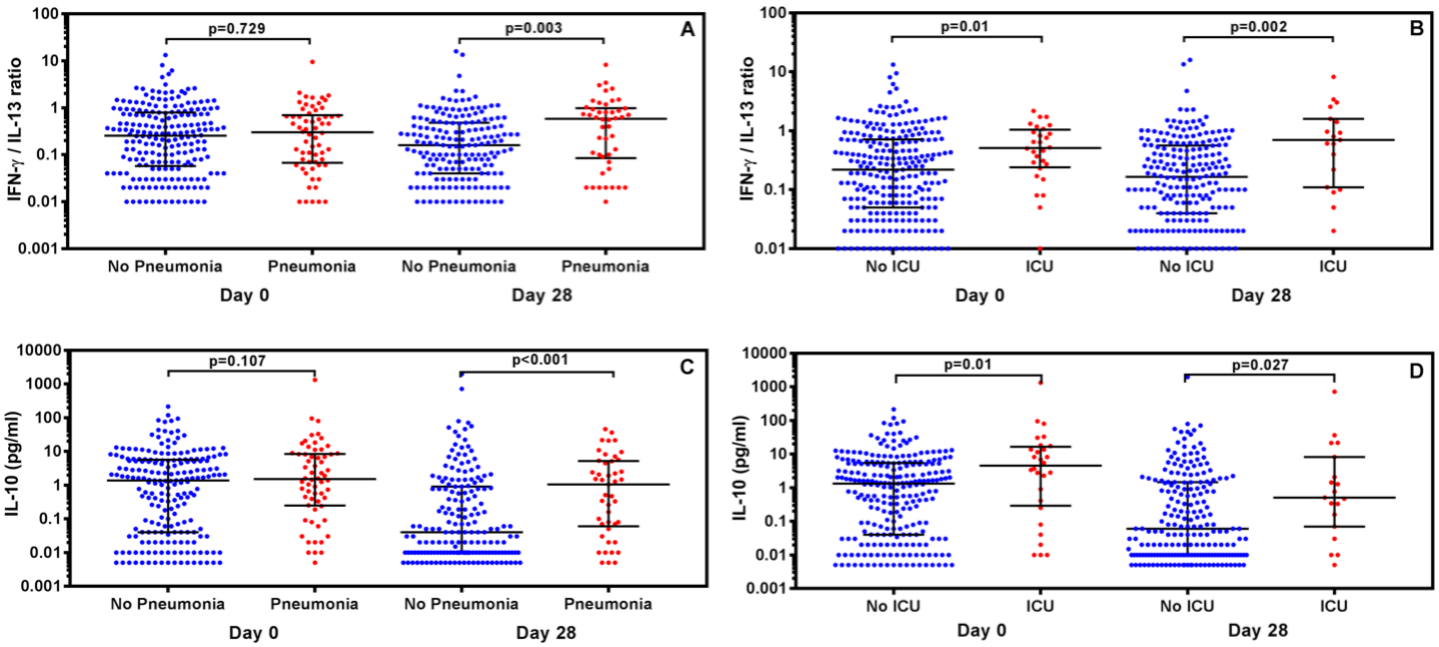
There was no difference in IFN-γ, IL-13 or IFN-γ/IL-13 ratio at day 0 and day 28 between patients who did or did not receive the influenza vaccine the season they became infected. Moreover, IL-10 levels at day 0 did not significantly differ between the two groups (data not shown). However, median IL-10 levels at day 28 were significantly lower in immunized patients (0.03pg/ml [IQR 0.01-0.93] vs 0.2pg/ml [IQR 0.03-2.40]; p=0.002).

Table 1. Demographics of patients infected with influenza.

Baseline demographics	**n=277**
Median age (IQR), yrs	56.0 (44.0-65.0)
Male/Female ratio	1.47 / 1
Median time since transplant (IQR), yrs	3.09 (0.67-9.44)
Transplantation	
Solid Organ Transplantation, n (%)	**224 (80.9)#**
kidney	*135 (60.2)*
liver	*34 (15.2)*
lung	*30 (13.4)*
heart	*23 (10.3)*
small bowel	*1 (0.4)*
pancreatic islets	*1 (0.4)*
Hematopoietic Stem Cell Transplantation, n(%)	**54 (19.5)**
allogeneic	*40 (74.1)*
autologous	*14 (25.9)*
Influenza infection	
Immunization for current influenza season, n (%)	170 (67%)*
Influenza Type	
Influenza A	187 (67.5)
H1	*80 (42.8)*
H3	*82 (43.9)*
unspecified	*23 (12.3)*
Influenza B	52 (18.8)
unknown	35 (12.6)
Complications	
Pneumonia	62 (22.4)
ICU admission	28 (10.1)
Death at 28 days	9 (3.2)
Death at 6 months	18 (6.5)

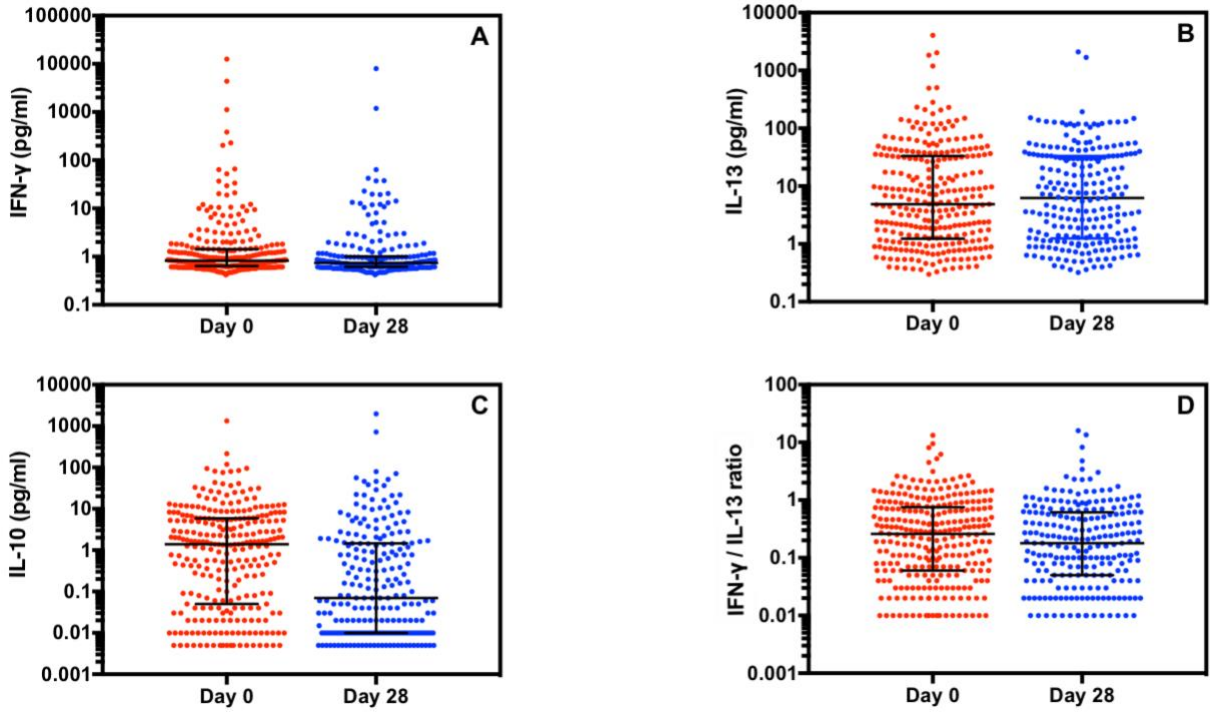
*information was not available in 23 patients. #: one patient had both allogenic stem cell and liver transplantation. IQR: interquartile range; ICU: intensive care unit.

Figure 1. Dot plots showing IFN-γ / IL-13 ratio as well as IL-10 levels at day 0 and 28 according to presence or absence of pneumonia and ICU admission



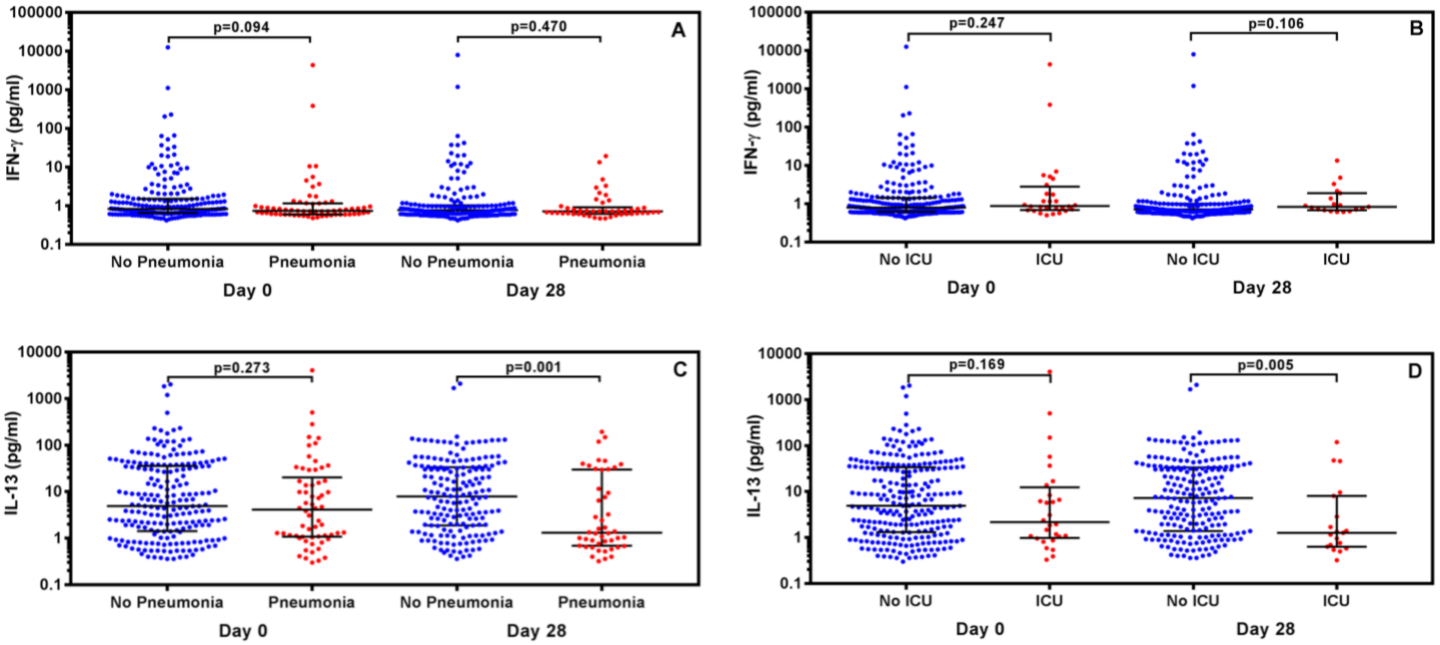
IFN: Interferon; IL: Interleukin; ICU:Intensive care unit
P-values <0.0125 were considered significant

Supplementary Figure 1. Distribution of IFN-γ, IL-13, IL-10 values and IFN-γ/IL-13 ratio in patients at day 0 and day 28



IFN: Interferon; IL: Interleukin

Supplementary Figure 2. Dot plots showing IFN-γ and IL-13 levels at day 0 and 28 according to presence or absence of pneumonia and ICU admission



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